XXXIII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

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(*Received March 20th, 1923.*)

Among the esters of phosphoric acid possessing biochemical interest three are known in which the phosphoric acid group is believed to be combined with a hexose molecule.

Hexosediphosphoric acid was discovered by Harden and Young [1905] and by Ivanov [1905] among the products of the fermentation of glucose, fructose or mannose by yeast juice in presence of phosphates, and was shown by Young [1909, 1911] to possess the constitution $C_6H_{10}O_4(PO_4H_2)_2$ and to yield fructose on hydrolysis. One of the two phosphoric acid molecules is very easily removed by boiling the ester with weak acids and in this way Neuberg [1918] obtained a hexosemonophosphoric acid $C_6H_{11}O_5(PO_4H_2)$ which like the diphosphoric ester is very slightly dextrorotatory and yields a laevorotatory sugar on further hydrolysis.

The presence of a hexosemonophosphoric ester was observed by Harden and Robison [1914] among the products of fermentation of sugar by yeast juice, and the isolation and investigation of this ester has been described by Robison [1922] in a recent number of this Journal. It is entirely different from the compound prepared by Neuberg, being strongly dextrorotatory and yielding a dextrorotatory sugar on hydrolysis. The phosphoric acid group in this ester is moreover much more slowly removed by boiling with acids than either of the groups in hexosediphosphoric acid.

The barium and calcium salts of the diphosphoric ester are sparingly soluble in cold and still less soluble in hot water. Those of the monophosphoric esters are on the contrary very easily soluble in water.

In animal physiology hexosediphosphoric acid has acquired great interest and significance through the researches of Embden and his co-workers who have brought forward convincing evidence [Embden and Lacquer, 1921] that this or some closely related phosphoric ester is present in striped muscle and is identical with the "lactacidogen."

During my investigation of the hexosemonophosphoric acid isolated from the products of fermentation, the hydrolysis of the ester by enzymes was studied. In some experiments in which the readily soluble calcium and barium

salts were used as substrate, the progress of the hydrolysis was shown by the formation of a precipitate of sparingly soluble calcium or barium phosphates,

$$C_6H_{11}O_5PO_4Ca + H_2O \rightarrow C_6H_{12}O_6 + CaHPO_4.$$

The formation of this precipitate suggested to me the query whether some such reaction might conceivably be concerned in the deposition of calcium phosphate during the formation of bone in the animal body. In the first instance I sought for the presence of an enzyme capable of effecting such hydrolysis in the bones of growing animals.

Experiment 1. *Aqueous extract of bone of rabbit.* The femur taken from a young rabbit immediately after death was pounded with water saturated with toluene and after two hours the extract was filtered. 0·1 cc. of this extract, equivalent to 0·02 g. fresh bone was incubated at 37° with 2 cc. of a 1 % solution of barium hexosemonophosphate + 0·05 cc. toluene. Controls containing (1) 0·1 cc. extract in 2 cc. water, (2) 2 cc. barium hexosemonophosphate solution without extract, were incubated at the same time. Within 18 hours a precipitate of barium phosphate had formed in the solution containing the phosphoric ester + bone extract but not in either of the controls. After 60 hours at 37° the free phosphate in all three tubes was estimated, the results indicating that 35 % of the total phosphoric acid had been hydrolysed by the bone extract.

The free phosphoric acid was estimated by precipitation with ammonium molybdate as in Neumann's method, omitting the previous ashing with nitric and sulphuric acid. The exact method employed was as follows:—A solution containing 5 cc. concentrated sulphuric acid, 25 cc. of a 50 % solution of ammonium nitrate, and 60 cc. water is heated to 75°–80°. The phosphate solution to be estimated is then added, followed immediately by 20 cc. of a 10 % solution of ammonium molybdate. If the volume of the phosphate solution is much greater than 10 cc. the amount of water is correspondingly reduced. The temperature of the solution before the addition of the ammonium molybdate should be 70°–75°. The flask is vigorously shaken and allowed to stand for $\frac{1}{4}$–$\frac{1}{2}$ hr. The yellow precipitate is then filtered off in the special funnel described by Plimmer and Bayliss [1906], washed with ice water and estimated in the usual way with standard NaOH solution, 0·1*N* solution being employed when the amount of free phosphoric acid corresponds with less than 2 mg. P. Carbon dioxide must be expelled by adding a slight excess of acid and boiling for a few minutes before the final titration.

With this method very varying quantities of phosphorus can be estimated with an accuracy of about 0·01 mg. (using 0·1*N* solution) or indeed, as Iversen [1920, 1, 2, 3] has shown, a still greater degree of accuracy may be obtained if special precautions are taken.

It cannot, of course, be taken for granted that no organic ester of phosphoric acid will be hydrolysed under the above conditions, but actual tests showed that with solutions of hexosemonophosphoric acid, glycerophosphoric

acid and even hexosediphosphoric acid, the amount of hydrolysis is negligible provided the temperature is not allowed to rise or the time prolonged unduly.

The total phosphorus was estimated in all cases by Neumann's method.

Experiment 2. *Bones of a young rat* (age 16 days, weight 28 g.). The epiphyseal cartilage was removed from both ends of each bone. The cartilage and shaft from one femur, tibia and humerus were each separately incubated 22 hours at 37° with 1 cc. of a solution of potassium hexosemonophosphate (sol. A) + 0·05 cc. chloroform. The cartilage and shaft of the second femur and tibia and the shaft of the humerus were incubated with 1 cc. water + 0·05 cc. chloroform. The cartilage of the second humerus was heated to 100° for a few minutes and then incubated with solution A. The weight of bone or cartilage in each tube was not accurately determined but was about 0·05 g.

Experiment 3. *Bones of a rachitic rat.* These were treated as described above—cartilage and shaft being separately tested.

The results of experiments 2 and 3 are shown in Table I.

Table I.

Experiment	Total P mg. P in 1 cc. sol. A	Free PO_4 after 22 hours at 37° (mg. P)				Percentage of combined P hydrolysed by tissue
		1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	
2. Normal rat:						
Femur shaft	3·53	0·10	0·00	1·76	1·66	48
„ cartilage	„	„	0·00	1·16	1·06	31
Tibia shaft	„	„	0·00	1·86	1·76	51
„ cartilage	„	„	0·00	1·35	1·25	36
Humerus shaft	„	„	0·00	1·27	1·17	34
„ cartilage	„	„	—	1·33	1·23	36
Humerus cartilage previously heated to 100°	„	„	—	0·09	−0·01	0
3. Rachitic rat:						
Humerus shaft	3·72	0·10	0·03	2·60	2·47	68
„ cartilage	„	„	0·07	2·95	2·78	77

These results clearly demonstrate the presence of an enzyme which can effect the hydrolysis of hexosemonophosphoric acid in the bones and ossifying cartilage of young rats and certainly not less in those of rachitic than those of normal animals. The next step was to discover whether the enzyme is present in other tissues also. For this purpose a young rat from the same litter as those used in the previous experiments was taken.

Experiment 4. *Tissues of young rat* (age 30 days, weight 48 g.). Weighed quantities (0·05 g. ± 5 %) of the various tissues were removed with all precautions against bacterial contamination and were incubated with 1 cc. of potassium hexosemonophosphate solution + 0·05 cc. chloroform (series A) or with 1 cc. of water + 0·05 cc. chloroform (series B). After 46 hours at 37° the contents of each tube were diluted with 5 cc. distilled water and filtered through a small Buchner funnel, the tube and funnel being rinsed out three times with 5 cc. water. Free phosphate was estimated in the filtrate and washings. The results are given in Table II. In the case of the spleen and

trachea the weight of tissue available did not permit of 0·05 g. being taken and the amount of phosphoric acid formed by hydrolysis has therefore been multiplied by the appropriate factor in order that the figures (enclosed in brackets) may be compared with those for the other tissues.

Table II.

Tissue	Weight g.	Total P mg. in 1 cc. sol. A	Free PO_4 after 46 hours at 37° (mg. P) 1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	Percentage of combined P hydrolysed by tissue
Femur cartilage (epiphysis)	·05	3·72	0·16	0·10	3·47	3·21	90
Rib cartilage	·05	„	„	0·08	0·30	0·06	2
Trachea	·02	„	„	0·04	0·30	0·10 (0·25)	3 (7)
Liver	·05	„	„	0·06	0·62	0·40	11
Spleen	·04	„	„	0·06	0·45	0·23 (0·29)	7 (8)
Thymus	·05	„	„	0·28	0·50	0·06	2
Heart muscle	·05	„	„	0·07	0·55	0·32	9
Pancreas	·05	„	„	0·21	0·48	0·11	3
Kidney	·05	„	„	0·15	2·59	2·28	64

The figures in the last column give the percentage of combined phosphoric acid set free by the tissue enzyme and point to an almost unique position for ossifying as distinct from non-ossifying cartilage in respect of this enzyme. Of the other tissues, though slight hydrolysis was produced in all cases, only the kidney approached the femur epiphysis in enzymic power. The trachea and rib cartilage are particularly interesting as examples of non-ossifying cartilage, both showing a very low hydrolytic power. In this experiment the ribs themselves were not examined nor the costachondral junctions, the cartilage being specially taken at some distance from the latter, but in a later experiment the costachondral junctions were found to contain the enzyme in a high degree and to be entirely comparable with cartilage of other bones in this respect.

In all the above experiments salts of hexosemonophosphoric acid were employed as substrate. In the next the action of various tissues on salts of hexosemonophosphoric, hexosediphosphoric and of glycerophosphoric acid was compared.

Experiment 5. *Tissues of rabbit* (age under 4 weeks). The tissues were macerated with twice their weight of water containing 2 % of chloroform until a fairly uniform mixture was obtained. 0·3 cc. of this maceration mixture, equivalent to 0·1 g. of fresh tissue was placed in each of the following solutions:

Sol. 0. 2 cc. water + 0·1 cc. chloroform
Sol. 1. „ potassium hexosemonophosphate sol. (combined P = 5·47 mg.) + 0·1 cc. chloroform
Sol. 2. „ „ hexosediphosphate sol. (combined P = 4·00 mg.) + 0·1 cc. „
Sol. 3. „ „ glycerophosphate sol. (combined P = 6·58 mg.) + 0·1 cc. „

All solutions were incubated for 40 hours at 37° and were then filtered and the free phosphate estimated as described above. 2 cc. of sols. 1, 2 and 3 were also incubated with 0·1 cc. chloroform but without the addition of any tissue, as controls. The traces of free phosphate present in these solutions were not increased by the incubation, indicating that no appreciable hydrolysis takes

place in the absence of tissue under these conditions. To avoid multiplying the number of columns these small amounts have been subtracted from the corresponding totals and are not shown in the table (Table III). The amount of free phosphate arising from autolysis of the tissues is given in the first column, and this has also been subtracted from the totals in order to arrive at the amount of phosphoric ester hydrolysed by the tissue, as shown in the remaining columns.

Table III.

	Free phosphate after 40 hours' incubation at 37° (mg. P)				Percentage of phosphoric ester hydrolysed by tissue		
Tissue	Tissue + sol. 0 (autolysis)	Tissue + sol. 1 less P in controls	Tissue + sol. 2 less P in controls	Tissue + sol. 3 less P in controls	Sol. 1 potassium hexosemono-phosphate	Sol. 2 potassium hexosedi-phosphate	Sol. 3 potassium glycero-phosphate
Femur cartilage (epiphysis)	0·13	5·19	3·56	3·64	95	89	56
Rib cartilage	0·01	0·45	2·14	0·39	8	54	6
Trachea cartilage	0·11	—	1·31	—	—	33	—
Blood	0·03	0·02	0·01	0·53	0	0	8
Liver	0·15	1·19	2·62	1·54	22	66	24
Spleen	0·22	1·18	1·98	0·89	22	50	14
Pancreas	0·20	0·31	0·92	0·37	6	23	6
Muscle	0·17	0·08	1·98	0·15	1	50	2
Kidney	0·16	2·22	3·17	1·33	41	79	20

The figures for sol. 1 (potassium hexosemonophosphate) follow in general the same order as those in Table II, and show the same striking superiority of the epiphyseal cartilage over other tissues with respect to this enzyme. The hydrolysis of the ester by the femur cartilage is almost complete while the kidney, which is again second, only hydrolysed 41 %. Liver and spleen were relatively rather more active than in the previous experiment while with muscle and blood practically no hydrolysis was shown. A somewhat similar order is found in the figures for sol. 3 (potassium glycerophosphate) though the degree of hydrolysis is usually less than with hexosemonophosphate[1]. Whether the somewhat pronounced variations between the two sets of figures, *e.g.* in those for liver and blood, indicate that a different enzyme is involved, can only be decided by further experiments. The action of tissue enzymes on glycerophosphates has been investigated by Grosser and Husler [1912] who found that kidney and intestine effected complete hydrolysis in 24 hrs. at 37°, liver only 16 %, while spleen, blood and muscle were inactive. Plimmer [1913] obtained similar results with kidney and intestine, but found extracts of liver and pancreas to be inactive. In these as in my own experiments the glycerophosphate employed was the commercial preparation and probably contained both α and β varieties. The results obtained by these investigators and my own (including experiments not recorded in this paper) point to a variable but usually very low hydrolytic power for liver and liver extracts with respect to both glycerophosphoric acid and hexosemonophosphoric acid. The figures for sol. 2 (potassium hexosediphosphate) in Table III

[1] The difference in the rate of hydrolysis of potassium hexosemonophosphate and potassium glycerophosphate has not been confirmed in later experiments and may have been due to slight differences in the p_H of the two solutions. (Note added April 13th.)

differ considerably from those for the other esters and appear at first sight to show no striking variations among themselves. On closer examination however, one interesting fact emerges, that nearly all the tissues examined possess an enzyme capable of hydrolysing up to 50 % of the combined phosphorus—*i.e.* one of the two phosphoric acid groups. Thus muscle and rib cartilage, which are almost without action on hexosemonophosphoric acid and glycerophosphoric acid both hydrolyse approximately 50 % of the phosphorus in the diphosphoric ester. Only blood is entirely inactive. The hydrolysis of the second phosphoric acid group appears then to follow approximately the same order as that for hexosemonophosphoric acid. Obviously two different enzymes are here involved. The very different hydrolytic power of muscle with regard to the two phosphoric acid groups is significant in view of the close relationship between hexosediphosphoric acid and lactacidogen.

Tomita [1922, 1 and 2] has also investigated the action of animal tissues on the synthetic sucrose monophosphate, and on Neuberg's hexosemonophosphate and has found them to possess hydrolytic power diminishing in the following order: kidney, liver, spleen, pancreas, muscle.

Discussion of Results.

It has thus been shown that the ossifying cartilage of young rats and rabbits contains a very active enzyme which rapidly hydrolyses hexosemonophosphoric ester and also glycerophosphoric ester. The kidney also contains this enzyme but its hydrolytic power is considerably less, for equal weights of tissue, than that of ossifying cartilage. Other tissues examined, including non-ossifying cartilage from ribs and trachea, contain the enzyme in very much smaller degree or not at all.

The possible significance of these facts in processes of ossification may now be considered. The cause of the deposition of calcium phosphate in the growing bone and its non-deposition elsewhere has not been explained. It has been calculated [Adler, 1908] that an aqueous solution containing the same amounts of inorganic salts as are found in serum would deposit a mixture of calcium phosphate and carbonate. The increased solubility of these salts in presence of serum proteins has been given [Pauli and Samec, 1909] as an explanation of the non-formation of such a precipitate in blood while specific adsorption of ions by the cartilage has been suggested [Pfaundler, 1904] to explain the deposition of calcium salts in bone. It has been shown by many investigators that blood contains in addition to lipoid phosphorus and inorganic phosphate a certain amount of combined phosphoric acid which is readily hydrolysed by boiling with acids. Thus Greenwald [1916] found a small amount of such combined phosphoric acid (less than 1 mg. P per 100 cc.) in serum. Bloor [1918] found similar amounts of organic phosphate (10 % of the total phosphates) in plasma of normal men and women but much greater amounts (60–80 % of the total phosphates) in the corpuscles. Jones and Nye [1921] have obtained similar figures for corpuscles from the blood of children of both sexes but

think that the unknown phosphoric acid in plasma is negligible in amount. In a later paper McKellips, De Young, and Bloor [1921] give analyses of blood of very young infants and find that the amount of this unknown phosphoric acid in the plasma is much higher (average 2·2 mg. P per 100 cc.) than for adults.

From a consideration of the work of these investigators it appears very probable that some ester of phosphoric acid which is soluble in acids but insoluble in ether and which is not precipitable by magnesia mixture or by ammonium molybdate is present in small amounts in plasma and in relatively large amounts in the corpuscles. If this ester is hydrolysable by the enzymes of ossifying cartilage the result would be an increase of HPO_4'' ions in the cartilage. If a condition of equilibrium exists between solid calcium phosphate on the one hand and a solution containing un-ionised calcium phosphate, and calcium and phosphate ions on the other, any increase in the concentration of either of these ions will result in further deposition of the solid calcium phosphate.

I would therefore suggest that the hydrolysis of some phosphoric ester, the calcium salts of which are readily soluble in water, by the enzyme in the ossifying cartilage, with production of free phosphoric acid may possibly be a factor in the process of bone formation. Whether this ester is a hexose-monophosphoric acid can only be decided by its isolation and this I am attempting to carry out. In conjunction with Mr H. D. Kay, experiments have already been performed which indicate with considerable probability that the "acid soluble" organic phosphate of blood is hydrolysed by the ossifying cartilage of young animals. The problem is also being attacked in another way with promising results. A preliminary experiment may be briefly described.

Bones from a severely rachitic rat were split lengthwise and one-half of each immersed in a solution of calcium hexosemonophosphate—the other half being immersed in normal saline. Both were incubated at 37° for two days in presence of chloroform and were then removed, washed with distilled water and fixed in formalin. Sections of these bones were made and stained with silver nitrate. The half which had been incubated with saline showed the customary greatly enlarged zone of provisional calcification practically devoid of phosphate while the half bone which had been incubated with the calcium hexosemonophosphate solution showed an intense black stain in the matrix of the cartilage cells when treated with silver nitrate. This may conceivably have been due merely to adsorption of the ester, but it was noted that the deposit occurred only in and throughout the depth of the zone of provisional calcification and not in the proliferating cartilage as might have been expected if adsorption were the sole cause. Further experiments must decide this point. For the histological investigations, as well as for the supply of the rachitic bones and for advice on this aspect of the work I am very greatly indebted to the kindness of Dr H. Goldblatt to whom I would here express my thanks.

SUMMARY.

An enzyme is present in the ossifying cartilage of young rats and rabbits, which rapidly hydrolyses hexosemonophosphoric acid, yielding free phosphoric acid.

In respect of this enzyme the kidney is considerably less active (about 50 %) than an equal weight of epiphyseal cartilage. Other tissues contain the enzyme in a very much lower degree, muscle and blood being almost inactive.

Non-ossifying cartilage shows less than one-tenth of the hydrolytic power of ossifying cartilage.

The same tissues in approximately the same order also hydrolyse glycerophosphoric acid.

One of the two phosphoric acid groups of hexosediphosphoric acid is very readily hydrolysed by almost all tissues (including muscle and non-ossifying cartilage) except blood.

The possible significance of this enzyme in the process of ossification in the animal body is discussed and certain preliminary experiments in various directions are briefly described, by the further prosecution of which it is hoped to obtain more information on this subject.

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